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(54) Title: MODIFICATION OF POLYPEPTIDES

(57) Abstract

The invention relates to a process for producing polymer-polypeptide conjugates with reduced immunogenicity comprising the steps of: a) generating a polymer halogen formiate; and b) conjugating at least one polymer-halogen formiate to at least one attachment group on the polypeptide (Pep). The invention also relates to a polymer-polypeptide conjugate with reduced immunogenicity, an activated polymer for conjugating polypeptides (Pep), and the use of said process for reducing the immunogenicity of polypeptides used for pharmaceutical and/or industrial applications. Finally it is the object of the invention to provide the use of said polymer-polypeptide conjugate for a number of therapeutic and industrial purposes, such as the use in pharmaceuticals, personal care products and detergent compositions.

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Title: Modification of polypeptides

FIELD OF THE INVENTION

The present invention relates to a process for producing polymer-polypeptide conjugates with reduced immunogenicity, a polymer-polypeptide conjugate with reduced immunogenicity, an activated polymer for conjugating polypeptides, the use of said process for reducing the immunogenicity of polypeptides, and the use of said polymer-polypeptide conjugate for a number of purposes.

BACKGROUND OF THE INVENTION

Due to the strongly extended use of polypeptides, such as especially proteins and enzymes, for industrial and pharmaceutical purposes an increasing number of people are daily exposed to polypeptides. This exposure may inflict problems for people having an enhanced inclination for eliciting an immune response toward polypeptides or to people frequently in direct contact with relatively large amounts of polypeptides.

The above mentioned groups of people include people regularly using pharmaceuticals comprising polypeptides. This group makes up a great many people, as people having a chronical disease, such as diabetes, need to take pharmaceutical products up to several times a day.

It is known that polypeptides being intentionally introduced into the circulatory system (*i.e.* the blood stream) of humans and animals, *e.g.* in the form of a pharmaceutical composition or suspension, may inflict the risk of causing an immune response as a result of antibody (or immunoglobulin) formation, such as the formation of mainly IgG, but also IgM.

However, the way of challenge is believed to have an important impact on the immune system's response. In the case of the polypeptides *e.g.* being inhaled through the respiratory passage the normal response will be formation of the antibody IgE leading to an allergic response.

This type of immune response may also be caused by "industrial polypeptides", such as *e.g.* enzymes comprised in detergents, personal care products, including cosmetics and the like, which are not intended to enter the circulatory system of the body.

For more than two decades scientists all over the world have made efforts to develop a technology making it possible to eliminate the immunogenicity, allergenicity, and/or antigenicity of polypeptides for therapeutic use. Even though the "perfect" technology has not yet been developed, some fruitful results have been achieved.

Most of the developed technologies involve chemical or genetic modification of polypeptides leading to larger and/or heavier polypeptide molecules. It seems that the molecular weight has an influence on the immune system's response towards polypeptides. However, today no precise commonly accepted explanation of the advantageous effect of larger and/or heavier polypeptide molecules exists. Further, it is possible to find exceptions from the rule. Even though a polypeptide such as *e.g.* plant pollen is both large and heavy it is known to cause an immune response (specifically an allergic response) for certain people.

One of the technologies, which has shown good results on polypeptides for therapeutic uses, involves modification of polypeptides by means of covalent attachment of strands of polyalkylene oxides (PAO), such as polyalkylene glycol (PAG), to polypeptide molecules.

Also naturally occurring polymers such as the polysaccharides dextran and pullulan are known to be used for conjugating polypeptides.

In general such chemical attachment of polymers to polypeptides is recognized to lead to polypeptides having increased stability, increased resistance to proteolytic inactivation, reduced immunogenicity, antigenicity and/or allergenicity, prolonged lifetime in the bloodstream, and a low toxicity.

The polymer polyalkylene oxide (PAO), such as polyethylene glycol (PEG), and methoxypolyethylene glycol (mPEG), capped on one end with a functional group, reactive with amines on the polypeptide molecule, is often used.

To effect covalent attachment of polyalkylene glycol (PAG) to a polypeptide the hydroxyl end-groups of the polymer must first be converted into reactive functional groups. This process is frequently referred to as "activation", and the product is called "activated", "derivatized" or "functionalized". To obtain a selective and specific attachment of the polymer strand to the polypeptide a linker molecule having a suitable coupling group is normally used.

Various PAG activation methods are known. One of the first commonly used methods involved activating the hydroxyl end-group of PAG using cyanuric chloride (Abuchowski et al., (1977), J. Biol. Chem., 252, 3578). However this method is not suitable for modifying polypeptide for foods and pharmaceuticals as cyanuric chloride (2,4,6-trichloro-s-triazine) are toxic, and the activated PAG is non-specific, as it reacts with polypeptides having functional groups other that amines, such as free essential cysteine or tyrosine residues.

Other methods commonly used for activation of insoluble polymers include activation of functional groups with cyanogen bromide, periodate, glutaraldehyde, biepoxides, epichlorohy-

drin, divenylsulfone, carbodiimide, sulfonyl halides, trichlorotriazine etc. (see R.F. Taylor, (1991), "Protein immobilisation. Fundamental and applications", Marcel Dekker, N.Y.; S.S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking", CRC Press, Boca Raton; G.T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques". Academic Press, N.Y.). Some of the methods concern activation of insoluble polymers but are also applicable to activation of soluble polymers *e.g.* periodate, trichlorotriazine, sulfonylhalides, divenylsulfone, carbodiimide etc. The functional groups being amino, hydroxyl, thiol, carboxyl, aldehyde or sulfydryl on the polymer, and the chosen attachment group on the protein must be considered in choosing the activation and conjugation chemistry.

Additional methods for activation of polyalkylene oxide polymers can also be found in WO 94/17039, US patent no. 5,324,844, WO 94/18247, WO 94/04193, US patent no. 5,219,564, US patent no. 5,122,614, WO 90/13540 (Enzon), and US patent no. 5,281,698 (Cetus), and more WO 93/15189 (Veronese) and for conjugation between activated polymers and enzymes *e.g.* Coagulation Factor VIII (WO 94/15625), haemoglobin (WO 94/09027), oxygen carrying molecule (US patent no. 4,412,989), ribonuclease and superoxide dismutase (Veronese at al., (1985), App. Biochem. Biotech., 11, 141-45).

Summarized the polymer activation research has been concentrated on finding activated PAO-polymers: 1) being reactive under mild process conditions, 2) having a high degree of selectivity towards specific attachment groups on the polypeptide, and 3) which, during the conjugation process, releases only residues being non-toxic.

Poly(ethylene glycol) succinoyl-N-hydroxysuccinimide ester (SS-PEG) (Abuchowski et al., (1984), Cancer Biochem. Biophys., 7, 175-186) is an example of such an activated PAO releasing only non-toxic residues, which are readily separable from the PAO-

polypeptide conjugate. However the product has limited stability in aqueous media.

Prior art patents

US patent no. 4,179,337 discloses a process for conjugation of polyalkylene glycol (PEG) to polypeptides for therapeutic use, such as insulin and certain enzymes, all having a physiological activity. The preparations of conjugated polypeptides have reduced immunogenicity, antigenicity, and have further a prolonged lifetime in the bloodstream as compared to the parent polypeptides. The surplus amounts of polymers necessary to conjugate the polypeptide makes the method expensive.

WO 90/13540 (Enzon Inc.) concerns activated PEG derivatives, namely, poly(ethylene glycol) succinimide carbonates (SC-PEG), the bifunctional derivative of PEG, namely, poly(ethylene glycol-bis-succinimide carbonate (BSC-PEG) and heterobifunctional derivatives of PEG, which in one end have the succinimide carbonate group and in the other end have a group readily reacting with amino groups of proteins to afford PEG attachment through stable urethane linkages.

Furthermore, WO 94/13540 discloses a process for synthesis of SC-PEG via PEG-chloroformiate by treating PEG with phosgene ($O=C-Cl_2$). The resulting PEG-chloroformiate is then reacted with N-hydroxysuccinimide (HOSu) followed by triethylamine (TEA) to yield the desired activated SC-PEG. During protein modification SC-PEG only releases non-toxic material (N-hydroxysuccinimide).

GB patent no. 1,183,257 (Crook et al.) describes chemistry for conjugation of enzymes to polysaccharides via a triazine ring.

US patent no. 5,133,968 (Kanebo, LTD.) describes a modified protease linked to a polysaccharide via a triazine ring leading to a suppressing effect on antigenicity and dermal hypersensitivity. The employed polysaccharide has an average

molecular weight not less than 10 kDa. The modification rate for surface amino acid groups in the modified protease is not less that 30%.

EP 632 082 (Heyleclina) describes the preparation of activated (methoxy)poly(alkylene glycol) carbamate (C-(m)PEG) from (m)PEG via (m)PEG-chloroformiate. The described activated (m)PEG carbamate has a high degree of selectivity, as it reacts only with amine groups. In the application it is asserted that (m)PEG chloroformiate readily reacts with amines, alcohols, phenols, and carboxylic acids.

Summarized, the prior art discloses activated polymer-polypeptide conjugates and processes for conjugating polypeptides to a number of polymers, including polyalkylene glycols (PAG), such as polyethylene glycols (PEG), and polysaccharides, such as dextran and pullulan.

Said prior art polypeptide modification processes are quite cumbersome, and slow, and result in moderate process yields, as it is necessary to add a surplus amount of activated polymer to obtain suitable absolute process yields. Consequently the production of such conjugates is relatively expensive.

Therefore, it would be desirable to be able to obtain polymer-polypeptide conjugates with reduced immunogenicity using a more efficient process, which will reduce the cost.

SUMMARY OF THE INVENTION

It is the object of the present invention to provide polymer-polypeptide conjugates with reduced immunogenicity for a number of purposes, including pharmaceutical and industrial purposes.

The present inventors have found an advantageous process for producing polypeptides with reduced immunogenicity using less

process steps than expected on the basis of prior art.

In the first aspect the invention relates to a process for producing polymer-polypeptide conjugates with reduced immunogenicity, using a polymer as the starting material, comprising the steps of

a) generating a polymer halogen formiate, and

b) conjugating at least one polymer halogen formiate to at least one attachment group on the polypeptide.

Further the invention is directed towards an activated polymer capable of linking to attachment groups on a polypeptide by acylation. The activated polymer has the general structure:

$Poly(-O-C=O-Y)_m$

wherein
Y is a halogen or nitrile
m is an integer from 1 to 25, and
Poly defines a synthetic or a naturally occurring polymer.

In a preferred embodiment of the invention Poly is a synthetic polymer, such as a polyalkylene oxide (PAO). In this case the activated polymer has the general structure

$R1 - (O-R2)_a - (O-R3)_b - (O-R4)_c - O - C=O - Y$

wherein
R1 is hydrogen, methyl, hydroxyl or methoxy,
R2 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,
R3 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,
R4 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,

a is an integer between 1 and 1000,
b is an integer between 0 and 1000,
c is an integer between 0 and 1000, and
Y is a halogen or a nitrile.

In another preferred embodiment Poly is a naturally occurring polymer, such as the polysaccharides dextran and pullulan.

In another aspect the invention relates to polymer-polypeptide conjugates, wherein the polymer can be a synthetic or naturally occurring polymer having the structure:

$$Poly(- O - C{=}O - X)_m(-Pep)_z$$

wherein
m is an integer between 1 and 25,
Poly can be a synthetic polymer or a naturally occurring polymer,
z is an integer between 1 and 25,
Pep can be any polypeptide, and
X is a coupling group between the polymer (Poly) and a polypeptide (Pep) which has been formed by reaction with a polymer halogen formiate.

In a preferred embodiment of the invention the polymer moiety (poly) of the conjugate is a synthetic polymer, such as a polyalkylene oxide (PAO) having the general formula

$$(R1 - (O-R2)_a - (O-R3)_b - (O-R4)_c - O - C{=}O - X -)_n Pep$$

wherein
R1 is hydrogen, methyl, hydroxyl or methoxy,
R2 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,
R3 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,
R4 is an alkyl group, which may be straight, branched,

disubstituted, or unsaturated,
a is an integer between 1 and 1000,
b is an integer between 0 and 1000,
c is an integer between 0 and 1000,
n is an integer between 1 and 100, and
X is a coupling group between the polymer (Poly) and a polypeptide (Pep) which has been formed by reaction with a polyalkylene oxide halogen formiate.

In another preferred embodiment the Poly moiety of the conjugate is an naturally occurring polysaccharide, such as dextran or pullulan.

Finally the invention relates to the use of the above process for reducing the immunogenicity of polypeptides, which include polypeptides for use in pharmaceutical and industrial products.

DETAILED DESCRIPTION OF THE INVENTION

It is the object of the invention to provide a process for conjugating polymers and polypeptides to obtain products with reduced immunogenicity.

In the context of the present invention the term "polypeptides" includes proteins, peptides and/or enzymes for pharmaceutical or industrial uses.

Is is especially contemplated to introduce polypeptides into the circulatory system of the body of humans and/or animals, which may inflict an immunogenic response.

Also polypeptides used as ingredients in industrial products, such as detergents and personal care products, including cosmetics, are contemplated. However, it is believed that it is not very likely that such "industrial polypeptides" come into direct contact with the circulatory system of the body of

humans or animals, as such polypeptide (or products comprising such polypeptides, are not injected (or the like) into the bloodstream. Such industrial polypeptides have a much greater risk of inflicting an allergic response, as a consequence of inhalation through the respiratory passage.

The "circulatory system" of the body of humans and animals means, in the context of the present invention, the system which mainly consists of the heart and blood vessels. The heart delivers the necessary energy for maintaining blood circulation in the vascular system. The circulation system functions as the organism's transportation system, when the blood transports O_2, nutritious matter, hormones, and other substances of importance for the cell regulation into the tissue. Further the blood removes CO_2 from the tissue to the lungs and residual substances to *e.g.* the kidneys. Furthermore, the blood is of importance for the temperature regulation and the defence mechanisms of the body, which include the immune system.

The term "pharmaceutical polypeptides" is defined as polypeptides, including peptides, such as peptide hormones, proteins and/or enzymes, being physiologically active when introduced into the circulatory system of the body. Examples of "pharmaceutical polypeptides" will be mentioned below.

In the context of the present invention "industrial polypeptides" are defined as polypeptides, including peptides, proteins and/or enzymes, which are not to be introduced into the circulatory system of the body of humans and animals. Examples of such polypeptides, which are specifically contemplated, are polypeptides used in products such as detergents, household article products, agrochemicals, personal care products, such as cosmetics, toiletries, oral and dermal pharmaceuticals, composition use for processing textiles, compositions for hard surface cleaning, and compositions used for manufacturing food and feed etc.

As described above it is known to produce polymer-polypeptide conjugates, such as polyalkylene oxide-polypeptide conjugates and polysaccharide-polypeptide conjugates by acylating the polymer with at least one polypeptide.

More specifically polymer-polypeptide conjugates can be prepared by first generating a polymer chloroformiate by using phosgene (Cl_2-C=O) and thereafter reacting it with N-hydroxysuccinimide (HOSu) followed by triethylamine (TEA) to yield the desired activated polymer-derivative.

The above conjugation process leads to attachment of the activated polymer to amine groups on the polypeptide. However, this process is cumbersome, slow, and costly.

In the first aspect the present invention relates to a process for producing polymer-polypeptide conjugates using a polymer (Poly) as the starting material. The conjugation process comprises the steps of

a) generating a polymer halogen formiate, and

b) conjugating at least one polymer halogen formiate to at least one attachment group on the polypeptide.

In comparison to the corresponding prior art process mentioned above, at least one process step has been eliminated, and still a product with reduced immunogenicity is obtained.

Polymer

According to the present invention the "polymer" (Poly) includes synthetic polymers and naturally occurring polymers.

Examples of suitable synthetic polymers are polymers which can be selected from the group comprising polyalkylene oxide (PAO), including polyalkylene glycols (PAG), such as polyethylene glycols (PEG) or methoxypolyethylene glycols (mPEG), polypropylene

glycols (PPG), and poly-vinyl alcohol (PVA), poly-carboxylates, poly-(vinylpyrolidone) and poly-D,L-amino acids.

In a preferred embodiment of the invention the polymer is a polyalkylene oxide (PAO), such as an polyalkylene glycol (PAG) or methoxypolyalkylene glycol (mPAG).

More specifically the PAG may advantageously be an polyethylene glycol (PEG) or a methoxypolyethylene glycol (mPEG).

Examples of suitable naturally occurring polymers include polysaccharide, such as agarose, guar gum, inulin, starch, dextran, pullulan, xanthan gum, carrageenin, pectin, alginic acid hydrolysates of chitosan etc., and derivatives thereof including hydroxypropylcellulose, methylcellulose. ethylcellulose, carboxymethylcellulose.

Especially contemplated according to the invention are dextran and pullulan.

Polymers having a molecular weight (M_r) between 1 and 60 kDa may be used according to the invention. Preferred are polymers having a molecular weight (M_r) of between 1 kDa and 1000 kDa, such as between 25 and 500 kDa, or between 2 kDa and 35 kDa, especially between 2 kDa and 25 kDa.

Note that all polymer molecular weights mentioned in this application are average molecular weights.

Advantages of the invention

Polypeptides with reduced immunogenicity can be produced in a less cumbersome way and also faster by the use of a process of the invention in comparison to prior art processes. Consequently, the process costs are less in comparison to the cost of equivalent prior art processes.

According to the above described document EP 0 632 082-A1 (see page 19, line 41) mPEG chloroformiate readily reacts with a number of chemical groups.

This is a simplification, as it is only correct when the reaction takes place in organic solvent. In an aqueous reaction solution chloroformiates will mainly react with (and attach to) amine groups on the polypeptide. Reaction with (attachment to) other groups will be much less pronounced.

Therefore, when using the process of the invention for obtaining a product consisting of polymers conjugated to the amine groups of polypeptides, it is preferred that the reaction solvent is mainly aqueous. If a less specific attachment to the polypeptide is needed, the composition of the reaction solvent is less important.

Consequently, the process of the invention provides the possibility of obtaining a "designed" conjugated polymer-polypeptide product, as the conjugation product is dependant on the solvent in which the conjugation reaction is carried out.

The present inventors have developed the process of the invention especially for large scale processing of polypeptides. However, the advantages of the process will also be present when producing smaller amounts of polymer polypeptide conjugates.

Another advantage of the invention is that the prepared polypeptide-polymer conjugate has an improved stability in comparison to the parent polypeptide.

The process of the invention has all of the above mentioned advantages. Further, only a minimum of process steps are used to obtain polypeptides with reduced immunogenicity, in comparison to the corresponding prior art processes.

Assessment of Immunogenicity

"Immunogenicity" is a wider term than "antigenicity" and "allergenicity", and expresses the immune system's response to the presence of foreign substances. Said foreign substances are called immunogens, antigens and allergens depending of the type of immune response the elicit.

An "immunogen" may be defined as a substance which, when introduced into circulatory system of animals and humans, is capable of stimulating an immunologic response resulting in formation of immunoglobulin.

The term "antigen" refers to substances which by themselves are capable of generating antibodies when recognized as a non-self molecule.

Further, an "allergen" may be defined as an antigen which may give rise to allergic sensitization or an allergic response by IgE antibodies (in humans, and molecules with comparable effects in animals).

Assessment of the immunogenicity may be made by injecting animal subcutaneously to enter the immunogen into the circulation system and comparing the effect with the corresponding parent polypeptides

A number of *in vitro* animal models exist for assessment of the immunogenic potential of polypeptides. Some of these models give a suitable basis for hazard assessment in man. Suitable models include a mice model.

This model seek to identify the immunogenic response in the form of the IgG response in BALB/C mice being injected subcutaneously with modified and unmodified polypeptides.

Also other animal models can be used for assessment of the immunogenic potential.

In Example 6 it is shown that by using the process of the invention the immunogenicity of polypeptides modified according to the process of the invention is reduced.

A polypeptide having "reduced immunogenicity" according to the invention indicates that the amount of produced antibodies, *e.g.* immunoglobulin in humans, and molecules with comparable effects in specific animals, which can lead to an immune response, is significantly decreased, when introduced into the circulatory system, in comparison to the corresponding parent polypeptide.

For BALB/C mice the IgG response gives a good indication of the immunigenic potential of polypeptides.

Polypeptides

The polypeptide to be conjugated according to the invention may be of plant, animal or microbial origin, although the polypeptides preferably is of microbial origin, such as of bacterial or fungal origin.

In another embodiment of the invention the polypeptide is a protein or peptide having a biological activity, such as an anti-microbial activity.

In an embodiment of the invention the polypeptide can be a "pharmaceutical polypeptide such as insulin, ACTH, glucagon, somatostatin, somatotropin, thymosin, parathyroid hormone, pigmentary hormones, somatomedin, erythropoietin, luteinizing hormone, chorionic gonadotropin, hypothalmic releasing factors, antidiuretic hormones, thyroid stimulating hormone, relaxin, interferon, thrombopoietin (TPO) and prolactin.

In another embodiment of the invention the polypeptide is an enzyme used in pharmaceuticals or industrial products including an enzyme selected from the group of proteases, lipases,

transferases, carbohydrases, oxidoreductases, and phytases.

Polypeptides with a molecular weight in the range between about 10 kDa and 100 kDa are especially contemplated.

In the cases of the polypeptide having a biological or enzymatic activity, said activity is substantially maintained.

A "substantially" maintained activity is, in the context of the present invention, defined as an activity which is at least between 20% and 30%, preferably between 30% and 40%, more preferably between 40% and 60%, better from 60% up to 80%, even better from 80% up to about 100%, in comparison to the activity of the parent polypeptide.

Between 1 and 25 polymer molecules, preferably from 1 to 10 polymer molecules, are conjugated to each polypeptide molecule. This is significantly less than corresponding prior art techniques. Consequently the expense to polymer is reduced. To some extent it entails that the activity of the enzyme is substantially retained, as it is to be anticipated that the activity to a certain extent varies inversely with the number and the size of polymer conjugated to the polypeptide.

The activated polymer, such as an polyalkylene oxide halogen formiate generated in step a) is preferably a polyalkylene oxide chloroformiate, such as methoxypolyalkylene glycol chloroformiate, and may be generated by using phosgene.

In Example 1 and Example 2 the preparation of methoxypolyethylene glycol chloroformiate 5,000 (mPEG 5,000) and 15,000 (mPEG 15,000), respectively, is described.

According to the invention the halogen may be any other halogen, such as Br or F.

In step b) of the process of the invention the attachment

groups on the polypeptide can be any group selected from the group including amines, hydroxyls, alcohols, phenols, or carboxylic acids.

More specifically the polymer halogen formiate may, if desired, attach to the side chain of *e.g.* serine, threonine, tyrosine, lysine, arginine, aspartate, glutamate in the polypeptide chain.

Further the invention is directed towards an activated polymer capable of linking to at least one attachment group on a polypeptide by an acylation reaction. The activated polymer has the general structure:

$$Poly(-O-C=O-Y)_m$$

wherein
Y is a halogen or nitrile
m is an integer between 1 and 25, and
Poly defines a synthetic polymer or a naturally occurring polymer.

In a preferred embodiment the activated polymer is an polyalkylene oxide (PAO) or polysaccharide capable of linking to attachment groups on a polypeptide.

In a preferred embodiment of the invention the polymer moiety (Poly) of the activated polymer is a synthetic polymer, such as polyalkylene oxide (PAO). The this case the activated polymer has the generic structure

$$R1-(O-R2)_a-(O-R3)_b-(O-R4)_c-O-C=O-Y$$

wherein
R1 is hydrogen, methyl, hydroxyl or methoxy,
R2 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,

R3 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,
R4 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,
a is an integer between 1 and 1000,
b is an integer between 0 and 1000,
c is an integer between 0 and 1000, and
Y is a halogen or nitrile

The halogen Y may be either of Cl, Br, or F, although Cl is preferred.

In another preferred embodiment of the invention the polymer moiety (Poly) is one of the above mentioned naturally occurring polysaccharides.

Preferred are dextran and pullulan.

The activated polymer of the invention may preferably have a molecular weight in the range from about 1 kDa to 60 kDa, preferably from about 2 kDa to 35 kDa, especially from about 2 kDa to 25 kDa.

It is also the object of the invention to provide a polymer-polypeptide conjugate having the structure:

$$Poly(-O-C=O-X)_m(-Pep)_z$$

wherein
m is an integer between 1 and 25,
Poly can be a synthetic polymer or a naturally occurring polymer,
z is an integer between 1 and 25,
polypeptide can be any polypeptide, and
X is a coupling group between the polymer (Poly) and a polypeptide (Pep) which has been formed by reaction with a polymer halogen formiate.

In a preferred embodiment of the invention the polymer moiety (Poly) of the conjugate is a synthetic polymer, such as a polyalkylene oxide (PAO) having the general formula

$$(R1 - (O-R2)_a - (O-R3)_b - (O-R4)_c - O - C=O - X -)_n \; Pep$$

wherein

wherein R1, R2, R3, R4, a, b, c, and n are defined as above. X is a coupling group between the polymer (Poly) and a polypeptide (Pep).

In another preferred embodiment the polymer moiety (Poly) of the conjugate is a naturally occurring polysaccharide, such as dextran or pullulan.

The coupling group has been formed by reacting the activated polymer (polymer halogen formiate) of the invention with an attachment group on the polypeptide (Pep). Said attachment group is preferably an amine, but can be any group selected from the group of amines, hydroxyls, alcohols, phenols, and/or carboxylic acids on the polypeptide (Pep).

The attachment group may be the side chain of serine, threonine, tyrosine, lysine, arginine, aspartate, glutamate in the polypeptide chain.

Preferably the polypeptide conjugate of the invention is produced by the process of the invention.

In a preferred embodiment of the invention R2, R3 and R4 is CH_2-CH_2, $CH_2-CH-CH_3$, or $CH_2-CH_2-CH_2-CH_2$.

Activated polymers of the invention have a molecular weight (M_r) between 1 and 60 kDa may be used according to the invention. Polymers are preferred having a molecular weight (M_r) of between 2 kDa and 35 kDa, especially between 2 kDa and 25 kDa.

The polypeptide to be conjugated is preferably a protein or peptide having a biological activity or an enzyme as mentioned above.

A polypeptide conjugate of the invention has a total molecular weight in the range of 50 kDa to 250 kDa, preferably between 80 and 200 kDa.

A conjugate of the invention can be stored by freezing *e.g.* at about 18°C.

Compositions

The invention also relates to pharmaceutical or industrial compositions comprising at least one polymer-polypeptide conjugate of the invention.

The composition may further comprise other polypeptides/proteins/enzymes/peptides and/or ingredients normally used in *e.g.* pharmaceuticals, detergents, agrochemicals, personal care products, composition use for treating textiles, compositions used of hard surface cleaning, compositions used for manufacturing food, *e.g.* baking, and feed etc.

Examples of said polypeptides include enzymes/polypeptides from the group including proteases, lipases, oxidoreductases, carbohydrases, transferases, such as transglutaminases, antimicrobial polypeptides, and phytases.

In an embodiment of the invention the polypeptide/peptide is insulin, ACTH, glucagon, somatostatin, somatotropin, thymosin, parathyroid hormone, pigmentary hormones, somatomedin, erythropoietin, luteinizing hormone, chorionic gonadotropin, hypothalmic releasing factors, antidiuretic hormones, thyroid stimulating hormone, relaxin, interferon, thrombopoietin (TPO) and prolactin.

The invention also relates to the use of a conjugate of the invention or a composition of the invention in pharmaceuticals or indistrial products defined above.

Finally the invention relates to a method for reducing the immunogenicity of polypeptides by using the process of the invention. The polypeptide may be any of the above mentioned.

METHODS AND MATERIALS

Materials:
Methoxypolyethylene glycol 15.000 (mPEG from Shearwater)
Methoxypolyethylene glycol 5.000 (mPEG from Fluka)
Subtilisin Novo (from Novo Nordisk A/S)
Carezyme© core (from Novo Nordisk A/S)
Succinyl-Alanine-Alanine-Proline-Phenylalanine-para-nitroanilide (Suc-AAPF-pNP) Sigma no. S-7388, Mw 624.6 g/mole.

ELISA reagents:
Horseradish Peroxidase-conjugated anti-IgG and anti IgE (Serotex Ldt, oxford, England)
Streptavidin-horse radish peroxidase (Kirkegård & Perry 14-30-00; dilution 1:1000).

Solutions:
Coating Buffer (0.15M Phosphate buffer solution, PBS):

NaCl	8.00g
KCl	0.20g
K_2PO_4	1.04g
$KHPO_4$	0.32g

Add H_2O to 1000 ml. pH 7.2

Blocking Buffer

Coating Buffer	100 ml
Skimmilk powder	2.00g

Washing Buffer

Coating Buffer 100ml
Tween20, 20% 50µl

Dilution Buffer
Blocking Buffer 25ml
H_2O 75ml
Tween20, 20% 50ml

Substrate Buffer
$C_6H_5Na_3O_7, 2H_2O$ 20.60g
$C_6H_8O_7$, H_2O 6.30g
Add H_2O to 1000ml, pH 5.0 - 5.2

Stop-solution (1M H2SO4)
H_2O 942ml
H_2SO_4 , 98-100 % 54ml

Sodium Borate, borax (Sigma)
3,3-Dimethyl glutaric acid (Sigma)
$CaCl_2$ (Sigma)
Tresyl chloride (2,2,2-triflouroethansulfonyl chloride) (Fluka)
Tween 20: Poly oxyethylene sorbitan mono laurate (Merck cat no. 822184)
1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Fluka)
N-Hydroxy succinimide (Fluka art. 56480))
Phosgene (Fluka art. 79380)
Lactose (Merck 7656)
PMSF (Phenyl Methyl Sulfonyl Flouride) (Sigma)

Colouring substrate:
OPD: o-phenylene-diamine, (Kementec cat no. 4260)

Test Animals:
BALB/C mice (about 20 grams)(purchased from Bomholdtgaard, Ry, Denmark))

Equipment:

XCEL II (Novex)
ELISA reader (UVmax, Molecular Devices)
HPLC (Waters)
PFLC (Pharmacia)
Superdex-75 column, Mono-Q, Mono S from Pharmacia, SW.
SLT: Fotometer from SLT LabInstruments
Size-exclusion chromatograph (Spherogel TSK-G2000 SWG).
Size-exclusion chromatograph (Superdex 200, Pharmacia, SW)
Amicon Cell

Protease activity

Analysis with Suc-Ala-Ala-Pro-Phe-pNA:
Proteases cleave the bond between the peptide and p-nitroaniline to give a visible yellow colour absorbing at 405 nm.

Buffer: *e.g.* Britton and Robinson buffer pH 8.3
Substrate: 100 mg suc-AAPF-pNA is dissolved into 1 ml dimethyl sulfoxide (DMSO). 100 µl of this is diluted into 10 ml with Britton and Robinson buffer.

Analysis
The substrate and protease solution is mixed, and the absorbance is monitored at 405 nm as a function of time and $ABS_{405\ nm}$/min. The temperature should be controlled (20-50°C depending on protease). This is a measure of the protease activity in the sample.

Analysis with Casein :
The analysis is performed according to standard Novo Nordisk procedures described in AF 220 which is hereby included as reference (and available of request).

Carezyme® activity
Enzymatic activity was measured as release of blue dye from azurine-crosslinked HE-cellulose (Cellazyme-C®). The reaction

was carried out at 40°C in 20 mM Na-phosphate pH 7 for 10 minutes. Release of dye was monitored by reading the absorbance at 595 nm in a UVmax® Elisa-reader. In addition, cellulytic activity was measured as described in "EAL-SM-0373.01/01" (available from Novo Nordisk on request).

ELISA IgG procedure for determine relative concentrations of IgG antibodies.

1) Coat the ELIAS-plates with 1µg protein/ml in coating buffer Incubate over night at 4°C, or at least 3 hours at room temperature. 50 µl/well. Shake gently.

2) Empty the plates and block with blocking buffer at least ½ hour at room temperature. 200 µl/well. Shake gently.
Wash the plates 3 times with Washing Buffer.

3) Antigen is incubated with ½ dilutions of sera in Dilution Buffer. Make those solutions just before adding them to the wells.
Keep some wells free for Dilution Buffer only (Blanks)
Incubate at least 1 hour at room temperature. 50 µl/well. Shake gently.
Wash the plates 3 times in Washning buffer.

4) Dilute Tracer (Horseradish Peroxidase-conjugated anti IgG and anti IgE) in Dilution Buffer. Incubate at room temperature at least 1 hour. 50 µl/well. Shake gently.
Wash the plates 3 times in Washing Buffer.

5) Mix 0.6 mg ODP/ml + 0.4 µl H_2O_2/ml in ubstrate Buffer. Make the solution just before use. Incubate for 10 minutes. 50 µl/well.

6) To stop the reaction: add Stop Solution. 50 µl/well.

7) Read the plates at 492 nm with 620 nm as reference.

Data is calculated and presented in Lotus.

Immunisation of mice

BALB/C mice (20 grams) are immunised 10 times (intervals of 14 days) by subcutaneous injection of the modified or unmodified polypeptide in question, respectively by standard proceedures known in art.

Determination of the molecular weight

Electrophoretic separation of proteins was performed by standard methods using 4-20% gradient SDS poly acrylamide gels (Novex). Proteins were detected by silver staining. The molecular weight was measured relative to the mobility of Mark-12® wide range molecular weight standards from Novex.

EXAMPLES

EXAMPLE 1

Preparation of mPEG 15,000 chloroformiate

10 gram mPEG 15,000 was suspended in 60 ml toluene of which 15 ml was distilled off to remove any trace of water. After cooling to ambient temperature some precipitate formed, which re-dissolved upon addition of 10 ml anhydrous dichloromethane. 1.7 ml phosgene (1.93 M in toluene 5 eqv.) was added without any detectable reaction. After 14 hours at ambient temperature the mixture was evaporated to dryness to remove phosgene. To improve the crystalline structure it is preferable to re-crystallise from toluene (dry 5 ml/ g mPEG). Yield after filtration and drying generally exceeds 98% and activation degree is better than 90% by NMR. ^{1}H-NMR for mPEG 15,000 ($CDCl_3$) δ 3.38 s (I= 2.6 CH_3 i OMe), 3.40* dd (I = 4.5%, ^{13}C

satellite), 3.64 bs (I = 1364 main peak), 3.89* dd (I = 4.8% , ^{13}C satellite), 4.46 q* (I = 1.8, CH_2 α to chloroformiat). When stored in an desiccator at 22°C, a decrease in activation degree of 40% was detected after 3 months and a new peak was detected at δ 4.37. When stored for 5 months a decrease of 70% was detected and the same peak at δ 4.37 was seen. When stored at -18°C no change was detected after 3 months.

EXAMPLE 2

Preparation of mPEG 5,000 chloroformiate

10 gram mPEG 5,000 was suspended in 60 ml toluene of which 15 ml was distilled off to remove any trace of water. After cooling to ambient temperature some precipitate formed, which redissolved upon addition of 10 ml anhydrous dichloromethane. 15.2 ml phosgene (1.93 M in toluene 5 eqv.) was added without any detectable reaction. After 14 hours at ambient temperature the mixture was evaporated to dryness to remove phosgene. To improve the crystalline structure the mixture was re-crystallised.

EXAMPLE 3

Conjugation of protease with mPEG 15,000-chloroformiate

To a solution (10 ml) of 100 mg of highly purified Subtilisin Novo in 0.1 M Borate (pH 9.5, 0.5 M NaCl) 3 x 550 mg of methoxypolyethylene glycol-15,000-chloroformiate was added sequential at 0, 30 & 75 minutes.

The resulting Subtilisin Novo-mPEG-15,000 conjugate was purified by size-exclusion chromatography using a Superdex-75 column in an HPLC-system.

The residual activity of the conjugate was assessed by using

suc-AAPF-pNP and casein as substrates:

- peptide substrate : 95%
- CM-casein : 60%

EXAMPLE 4

Conjugation of a protease with mPEG 5,000 chloroformiate

To a solution (10 ml) of 100 mg of highly purified Subtilisin Novo in 0.1 M Borate (pH 9.5) and 50% dimethylformamide (DMF) 3 x 367 mg of methoxypolyethylene glycol 5,000 chloroformiate was added sequentially at 0, 30 & 75 minutes.

The reaction was performed at ambient temperature using magnetic stirring. At time 120 minutes the reaction was terminated by addition of 0.5 ml of 2 M Glycine.

The resulting Subtilisin Novo-mPEG-5,000 conjugate was purified by size-exclusion chromatography using a Superdex-75 column in an HPLC-system.

The residual activity of the conjugate is assessed by using suc-AAPF-pNP as substrate. The conjugated retained activity towards the peptide substrate.

EXAMPLE 5

Conjugation of a cellulase with mPEG 5,000 chloroformiate

The catalytic core-domain of Carezyme© was prepared according to Boisset, C. et al. (1995), FEBS Lett. 376, p. 49-52.

To a solution (12 ml) of 100 mg of the purified Carezyme© core in 0.1 M $NaHCO_3$, 0.2 M Na-Borate (pH 8.5) 1.5 g of methoxy-polyethylene glycol 5,000 chloroformiate was added.

The reaction was performed at ambient temperature using magnetic stirring. At time 30 minutes the reaction was

terminated by addition of 1 ml of 2 M Glycine.

The resulting Cellulase-core-domain-mPEG-5,000 conjugate was purified by desalting in 0.05 M $NaHCO_3$ and concentrated to the original volume using an Amicon Cell.

The obtained conjugate was PEGylated a second time using 1.0 g of methoxypolyethylene glycol 5,000 chloroformiate at otherwise identical conditions.

The final two times PEGylated Carezyme© core-mPEG-5,000 conjugate was purified by desalting in water and concentrated to approximately 1 mg/ml of protein using an Amicon Cell. The conjugate was stored at 4°C.

EXAMPLE 6

Immunogenecity assessment in mice

To assess the immunogenic potential of polypeptides modified according to the process of the invention Carezyme© core (unmodified), Carezyme© core-PEG 5,000 (modified, Example 5), Subtilisin Novo (unmodified), and Subtilisin Novo-PEG 15,000 (modified, Example 3), 0.9% NaCl (control) was injected subcutaneously into BALB/C mice.

Sera from immunized mice were tested in the ELISA assay (described above) to elucidate whether the molecules had activated the immune response system giving rise to a IgG response.

The results of the tests are shown in Table 1 and 2

Table 1

Specific IgG	Range	Median (10 mice)
NaCl (control)	0,155 - 0,388	0,177
Sub.Novo	0,130 - 1,379	0,865
Sub.Novo-PEG 15,000	0,218 - 0,519	0,333

Table 2

Specific IgG	Range	Median (10 mice)
NaCl (control)	0.016 - 0.140	0.034
Carezyme© core	1.841 -1.932	1.877
Carezyme core-mPEG 5,000	0.31 - 0.229	0,044

As can be seen from the Table 1 and 2 the amount of IgG response of BALB/C mice exposed to polypeptides modified according to the invention the immunogenicity have been reduced significantly in comparison to the mice having been exposed to the parent unmodified polypeptides.

As will be apparent to those skilled in the art, in the light of the foregoing disclosure, many alterations and modifications are possible in the practice of this invention without departing from the spirit or scope thereof. Accordingly, the scope of the invention is to be construed in accordance with the substance defined by the following claims.

PATENT CLAIMS

1. A process of producing polymer-polypeptide conjugates with reduced immunogenicity using a polymer (Poly) as the starting material, comprising the steps of

a) generating a polymer halogen formiate, and

b) conjugating at least one polymer halogen formiate to at least one attachment group on the polypeptide (Pep).

2. The process according to claim 1, wherein the polymer (Poly) is a synthetic polymer.

3. A process according to claim 2, wherein the synthetic polymer is selected from the group comprising polyalkylene oxide (PAO), including polyalkylene glycols (PAG), such as polyethylene glycols (PEG) or methoxypolyethylene glycols (mPEG), polypropylene glycols (PPG), and poly-vinyl alcohol (PVA), poly-carboxylates, poly-(vinylpyrolidone) and poly-D,L-amino acids.

4. The process according to claim 3, wherein the polymer (Poly) is a polyalkylene oxide (PAO), such as a polyalkylene glycol (PAG) or a methoxypolyalkylene glycol (mPAG).

5. The process according to claim 4, wherein the PAG or mPAG is a polyethylene glycol (PEG) or methoxypolyethylene glycol (mPEG).

6. The process according to claim 1, wherein the polymer (Poly) is a naturally occurring polymer.

7. The process according to claim 6, wherein the naturally occurring polymer is a polysaccharide selected from the group comprising agarose, guar gum, inulin, starch, dextran, pullulan, xanthan gum, carrageenin, pectin, alginic acid

hydrolysates of chitosan, and derivatives thereof.

8. The process according to claims 7, wherein the polysaccharide is dextran or pullulan.

9. The process according to any of claims 1 to 8, wherein the polypeptide (Pep) is a protein or a peptide.

10. The process according to any of the claims 1 to 8, wherein the polypeptide has anti-microbial activity.

11. The process according to any of claims 1 to 10, wherein the protein or peptide are physiologically active.

12. The process according to claim 11, wherein the protein or peptide being physiologically active is selected from the group of insulin, ACTH, glucagon, somatostatin, somatotropin, thymosin, parathyroid hormone, pigmentary hormones, somatomedin, erythropoietin, luteinizing hormone, chorionic gonadotropin, hypothalmic releasing factors, antidiuretic hormones, thyroid stimulating hormone, relaxin, interferon, thrombopoietin (TPO) and prolactin.

13. The process according to any of claims 9 to 11, wherein the protein is biologically active.

14. The process according to any of claims 9 to 13, wherein the protein has enzymatic activity.

15. The process according to claim 14, wherein the enzyme is a protease, a lipase, a transferase, a carbohydrase, an oxidoreductase, or a phytase.

16. The process according to any of claims 1 to 15, wherein the polymer halogen formiate is a polyalkylene oxide chloroformiate.

17. The process according to claim 16, wherein the polymer chloroformiate is a polyalkylene oxide chloroformiate, such as a polyalkylene glycol chloroformiate.

18. The process according to claim 17, wherein the polyalkylene glycol chloroformiate is a polyethylene glycol chloroformiate.

19. The process according to claim 18, wherein the polyethylene glycol chloroformiate is methoxypolyethylene glycol chloroformiate.

20. The process according to any of claims 1 to , wherein the polymer halogen formiate is a polysaccharide chloroformiate.

21. The process according to any of claims 1 and 6 to 8, wherein the polysaccharide chloroformiate is a dextran chloroformiate.

22. The process according to any of claims 1 to 21, wherein step a) is mediated by phosgene (Cl_2-C=O).

23. The process according to any of claims 1 to 22, wherein the attachment groups are at least one selected from the groups including amines, alcohols, phenols, or carboxylic acids.

24. The process according to claim 23, wherein the polymer halogen formiate attaches to the side chain serine, threonine, tyrosine, lysine, arginine, aspartate, glutamate in the polypeptide chain.

25. An activated polymer capable of linking to attachment groups on a polypeptide (Pep), which activated polymer has the general structure

Poly(- O - C=O - Y)$_m$

wherein

Y is a halogen or nitrile
m is an integer from 1 to 25, and
Poly defines a synthetic polymer or a naturally occurring polymer.

26. The activated polymer according to claim 25, wherein the polymer (Poly) is a synthetic polymer.

27. The activated polymer according to claims 26, wherein the polymer (Poly) is a polyalkylene oxide (PAO), having the general structure

$$R1 - (O-R2)_a - (O-R3)_b - (O-R4)_c - O - C=O - Y$$

wherein
R1 is hydrogen, methyl, hydroxyl or methoxy,
R2 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,
R3 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,
R4 is an alkyl group, which may be straight, branched, disubstituted, or unsaturated,
a is an integer between 1 and 1000,
b is an integer between 0 and 1000,
c is an integer between 0 and 1000, and
Y is a halogen or nitrile

28. The activated polymer according to claim 27, wherein the halogen is Cl, Br, or F.

29. The activated polymer according to claims 27 and 28, wherein R2, R3 and R4 is CH_2-CH_2, $CH_2-CH-CH_3$, or $CH_2-CH_2-CH_2-CH_2$.

30. The activated polymer according to claim 25, wherein the polymer (Poly) is a naturally occurring polysaccharide selected from the group defined in claim 7.

31. The activated polymer according to claim 30, wherein the naturally occurring polysaccharide is dextran or pullulan.

32. The activated polymer according to any of claims 25 to 31, wherein the molecular weight lies between 1 kDa and 60 kDa, preferably between 2 kDa and 35 kDa, especially between 2 kDa and 25 kDa.

33. A polymer-polypeptide conjugate having the general formula

$$Poly(- O - C=O - X)_m(-Pep)_z$$

wherein
m is an integer between 1 and 25,
Poly can be a synthetic polymer or a naturally occurring polymer,
z is an integer between 1 and 25,
Pep can be any polypeptide, and
X is a coupling group between the polymer (Poly) and a polypeptide (Pep) which has been formed by reaction with a polymer halogen formiate.

34. The polymer-polypeptide conjugate according to claim 33, wherein the polymer moiety (Poly) is a synthetic polymer.

35. The polymer-polypeptide conjugate according to claim 34, wherein the polymer (poly) is a polyalkylene oxide (PAO) having the general formula

$$(R1 - (O-R2)_a - (O-R3)_b - (O-R4)_c - O - C=O - X -)_n Pep$$

wherein
R1, R2, R3, R4, a,b,c is defined in claim 27,
n is an integer between 1 and 100, and
X is a coupling group between the polymer (poly) and a polypeptide (Pep).

36. The polymer-polypeptide conjugate according to claim 33, wherein the polymer moiety (Poly) is a naturally occurring polysaccharide.

37. The polymer-polypeptide conjugate, according to claim 36, wherein the naturally occurring polysaccharide is defined in claim 7.

38. The conjugate according to any of claims 33 to 37 being produced by the process according to claims 1 to 24.

39. The conjugate according to any of claims 33 to 38, wherein X is an attachment group selected from the group including amines, alcohols, phenols, and/or carboxylic acid group on the polypeptide.

40. The conjugate according to any of claims 35 to 39, wherein R2, R3 and R4 is CH_2-CH_2, CH_2-CH-CH_3, or CH_2-CH_2-CH_2-CH_2.

41. The conjugate according to any of claims 33 to 40, wherein the polypeptide (Pep) is a protein or peptide.

42. The conjugate according to any of claims 33 to 41, wherein the polypeptide (Pep) has anti-microbial activity.

43. The conjugate according to claim 41 and 42, wherein the protein has biological activity.

44. The conjugate according to any of claims 41 to 43, wherein the protein or peptide is physiologically active.

45. The conjugate according to claim 44, wherein the protein or peptide being physiologically active is selected from the group of insulin, ACTH, glucagon, somatostatin, somatotropin, thymosin, parathyroid hormone, pigmentary hormones, somatomedin, erythropoietin, luteinizing hormone, chorionic gonadotropin,

hypothalmic releasing factors, antidiuretic hormones, thyroid stimulating hormone, relaxin, interferon, thrombopoietin (TPO) and prolactin.

46. The conjugate according to any of claims 41 to 44, wherein the protein has enzymatic activity.

47. The conjugate according to claim 46, wherein the enzyme is a protease, a lipase, a transferase, a carbohydrase, an oxidoreductase, or a phytase.

48. The conjugate according to any of claims 33 to 47, wherein the total molecular weight lies between 50 kDa and 250 kDa, preferably between 80 kDa and 200 kDa.

49. The conjugate according to any of claims 33 to 48, wherein the molecular weight of the polymer lies between 1 kDa and 60 kDa, preferably between 2 kDa and 35 kDa, especially between 2 kDa and 25 kDa.

50. A composition comprising a polymer-polypeptide conjugate according to any of claims 33 to 49, which further comprises polypeptides/proteins/peptides/enzymes and/or ingredients normally used in pharmaceuticals or industrial products.

51. The composition according to claim 50, wherein the composition is a pharmaceutical composition.

52. The composition according to claim 50, wherein the pharmaceutical composition comprises an active ingredient selected from the group of insulin, ACTH, glucagon, somatostatin, somatotropin, thymosin, parathyroid hormone, pigmentary hormones, somatomedin, erythropoietin, luteinizing hormone, chorionic gonadotropin, hypothalmic releasing factors, antidiuretic hormones, thyroid stimulating hormone, relaxin, interferon, thrombopoietin (TPO) and prolactin.

53. The composition according to claim 50, wherein the composition used in an industrial product, such as a detergent or a personal care product.

54. Use of a conjugate according to any of claims 33 to 49 or a composition of any of claims 50 to 53 in pharmaceuticals or indistrial products.

55. A method for reducing the immunogenicity of polypeptides by using the process of any of claims 1 to 24.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00250

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 17/08, C07K 17/10
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

BIOSIS, MEDLINE, EMBASE, CA, CLAIMS, WPI, JAPIO

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 9607670 A1 (SURFACE ENGINEERING TECHNOLOGIES DIVISION OF INNERDYNE, INC.), 14 March 1996 (14.03.96), page 3, the claims --	1-55
X	EP 0557199 A1 (TRANSGENE S.A.), 25 August 1993 (25.08.93), page 2, line 38 - line 41, the claims --	1-55
X	DD 287951 A5 (AKADEMIE DER WISSENSCHAFTEN DER DDR), 14 March 1991 (14.03.91), the claims --	1-55

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 August 1996	02 -09- 1996
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Ake Lindberg Telephone No. +46 8 782 25 00

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00250

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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X	Dialog Information Services, File 155, Medline, Dialog accession no. 09093730, Medline accession no. 95023730, G.I. Shelykh et al: "Preparation and properties of trypsin modified by polysaccharides". Poluchenie i svoistva tripsina, modifitsirovannogo polisakharidami. Prikl Biokhim Mikrobiol (RUSSIA) Jan-Feb 1979, 15 (1) p82-7	1-55
X	EP 0632082 A1 (HEYLECINA, SOCIETE ANONYME), 4 January 1995 (04.01.95), page 11, the claims	1-55
X	WO 9013540 A1 (ENZON, INC.), 15 November 1990 (15.11.90), page 13, claim 7	1-55
X	WO 9101758 A1 (DEBIOPHARM S.A.), 21 February 1991 (21.02.91), example 1 and the claims	1-55
A	US 5133968 A (H. NAKAYAMA ET AL), 28 July 1992 (28.07.92), see whole document	1-55
A	WO 9404193 A1 (ENZON, INC.), 3 March 1994 (03.03.94), abstract, example 2 and the claims	1-55
A	WO 9417039 A1 (ENZON, INC.), 4 August 1994 (04.08.94), page 1, example 2	1-55

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00250

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DE 3440141 A1 (ECKERT, HEINER), 7 May 1986 (07.05.86), page 6, claims 1,7 --	1-55
A	US 4179337 A (F.F. DAVIS ET AL), 18 December 1979 (18.12.79), see whole document --	1-55

INTERNATIONAL SEARCH REPORT

Information on patent family members

31/07/96

International application No.
PCT/DK 96/00250

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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		ES-T-	2069082	01/05/95
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		CA-A-	2154170	04/08/94
		EP-A-	0681572	15/11/95
		JP-T-	8506131	02/07/96
		US-A-	5349001	20/09/94
		US-A-	5405877	11/04/95
DE-A1- 3440141	07/05/86	NONE		
US-A- 4179337	18/12/79	CA-A-	1033673	27/06/78
		CH-A-	616942	30/04/80
		DE-A,C-	2433883	05/02/76
		FR-A,B-	2313939	07/01/77
		GB-A-	1469472	06/04/77
		JP-C-	1152589	30/06/83
		JP-A-	50042087	16/04/75
		JP-B-	56023587	01/06/81
		NL-A-	7409770	22/01/75
		SE-B,C-	441753	04/11/85
		SE-A-	7409366	21/01/75